PCT / SE 2004 / 0 0 1 9 5 5

PRV

PATENT- OCH REGISTRERINGSVERKET
Patentavdelningen

Intyg
Certificate

Härmed intygas att bifogade kopior överensstämmer med de handlingar som ursprungligen ingivits till Patent- och registreringsverket i nedannämnda ansökan.

This is to certify that the annexed is a true copy of the documents as originally filed with the Patent- and Registration Office in connection with the following patent application.

(71) *Sökande* / *Applicant (s)* — *Biofol AB, Västra Frölunda SE*

(21) *Patentansökningsnummer* / *Patent application number* — *0303526-8*

(86) *Ingivningsdatum* / *Date of filing* — *2003-12-22*

Stockholm, 2004-12-28

För Patent- och registreringsverket
For the Patent- and Registration Office

Gunilla Larsson

Avgift
Fee

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

PATENT- OCH REGISTRERINGSVERKET
SWEDEN

Postadress/Adress
Box 5055
S-102 42 STOCKHOLM

Telefon/Phone
+46 8 782 25 00
Vx 08-782 25 00

Telex
17978
PATOREG S

Telefax
+46 8 666 02 86
08-666 02 86

AWAPATENT AB
Kontor/Handläggare
Göteborg/Inga-Lill Andersson/ILA

BIOFOL AB
Ansökningsnr

Vår referens
SE-21005894

CHEMOTHERAPEUTIC AGENTS

Technical field

The present invention relates to the use of a chemotherapeutic agent selected from the group consisting of multi-targeting antifolates, anthracyclines, platinum derivatives, topoisomerase inhibitors, and antimetabolites for the manufacture of a pharmaceutical composition for the treatment of cancer.

Background art

The control of drug-related toxicity (e.g. myelosuppression, diarrhea, mucosal toxicity, and infection) has been a major concern throughout the history of cancer chemotherapy. Substances conventionally used in cancer chemotherapy are e.g. antifolates, anthracyclines, and antineoplastic agents.

A widely used chemotherapeutic drug is doxorubicin (Adriamycin), belonging to the group of anthracyclines. Doxorubicin kills cells efficiently, but treatment is complicated by e.g. dose-limiting cardiotoxicity and multidrug resistance (Swift Lonnie P et al; "Activation of Adriamycin by the pH-dependent Formaldehyde-releasing Prodrug Hexamethylenetetramine", Molecular Cancer Therapeutics, Vol 2, 189-198, February 2003).

Oxaliplatin is another chemotherapeutic drug belonging to the group of platinum derivatives. Oxaliplatin is active as a single-agent as a first-line treatment for patients with advanced colorectal cancer (CRC). However, a common side-effect caused by oxaliplatin is polyneuropathy.

Irinotecan is another chemotherapeutic drug, a topoisomerase inhibitor, which is used to treat colon and rectal cancers. A common side-effect of such treatment is severe diarrhea.

Antifolates, or folate antagonists, constitute a class of antimetabolites, and are active chemotherapeutic agents for many solid tumor and hematologic malignancies (Thomas Purcell W et al; "Novel Antifolate Drugs", Evolving Therapies, pp 114-125, March 2003).

For many years, methotrexate (MTX) has been the major antifolate antimetabolic agent used in clinical medicine. The primary cellular target of MTX is the enzyme dihydrofolate reductase (DHFR) and thus, MTX is a single enzyme targeting antifolate. There are also other approved agents which only attack single enzyme targets, e.g. fluorouracil, which inhibit thymidylate synthase (TS) (Scagliotti, Giorgio V et al; Phase II Study of Pemetrexed With and Without Folic Acid and Vitamin B12 as Front-Line Therapy in Malignant Pleural Mesothelioma", Journal of Clinical Oncology, Vol 21, No 8, April 15 2003, pp 1556-1561).

Although response to treatment is observed in patients, many of them relapse due to development of resistance. Drug resistance is often a limiting factor in successful chemotherapy (Banerjee D et al; "Novel aspects of resistance to drugs targeted to dihydrofolate reductase and thymidylate synthase", Biochimica et Biophysica Acta 1587 (2002) 164-173).

Novel antifolates have been developed to improve the efficacy and toxicity profile or to decrease the various known mechanisms of resistance to antifolate therapy. These novel antifolates are multitargeting antifolates that have demonstrated broad-spectrum antitumor activity. This new generation of antifolates inhibits several key folate-requiring enzymes of the thymidine and purine biosynthetic pathways, in particular thymidylate synthase, DHFR and GARFT (glycinamide ribonucleotide formyltransferase), by competing with reduced folated for binding sites. The consequent inhibition of intracellular folate metabolism leads to the inhibition of cell growth (Niyikiza Clet et al; "Homocysteine and Methylmalonic Acid:

Markers to Predict and Avoid Toxicity from Pemetrexed Therapy", Molecular Cancer Therapeutics, Vol 1, 545-552, May 2002).

The cytotoxic activity and subsequent effectiveness of antifolates can be associated with substantial toxicity for some patients. Antifolates, as a class, have been associated with sporadic severe myelosuppression with gastrointestinal toxicity. A combination of such toxicities can carry a high risk of mortality. The inability to control these toxicities has led to the discontinuation of clinical development of some antifolates, and complicated the clinical development of others. (Niyikiza Clet et al; "Homocysteine and Methylmalonic Acid: Markers to Predict and Avoid Toxicity from Pemetrexed Therapy", Molecular Cancer Therapeutics, Vol 1, 545-552, May 2002).

US 5 376 658 (Spears et al) discloses the use of CF_2FH_4, and its solution product isomer FH_4, as a modulator of 5-fluorouracil in cancer chemotherapy. Also disclosed is a method of using CF_2FH_4 or FH_4 in order to reduce the toxicity of an anti-folate drug which has been administered to a patient. The anti-folate drugs disclosed are methotrexate, trimetrexate, nitrous oxide, and dideoxytetrahydrofolic acid.

All the above-mentioned anti-folate drugs belongs to the group of single enzyme targeting antifolates. However, until now, no satisfactory way of reducing the toxicity of multitargeting antifolates has been proposed. Considering the very promising action of antifolates, a possibility to efficiently reduce their side effects is very much sought-after.

Treatment with specific TS inhibitors like 5-FU in combination with folinic acid has been shown to reduce side-effects without reducing tumor effect. It seems that folate deficiency may have contributed to the toxicity in some cancer patients, and nutritional supplementation with folic or folinic acid had led to a reduction in toxicity and treatment-related deaths with preservation of

anticancer activity (Calvert H; "Folate status and the safety profile of antifolates"; Semin Oncol 2002;29:3-7). In fact, folates in combination with multifunctional antifolates e.g. pemetrexed may also lead to reduced side-effects.

In a recent study, Niyikiza et al reported that supplementation with folate may lead to a better safety profile in patients treated with pemetrexed, and possibly to an improved efficacy. Toxicity could be modulated by folic acid supplementation, and the maximum tolerated dose could be increased (Niyikiza C et al, "Homocysteine and methylmalonic acid: markers to predict and avoid toxicity from pemetrexed therapy"; Mol Cancer Ther 2002;1:545-52).

However, the metabolism of folic acid is a very complex process, and many metabolic steps are required in order to achive the active substances of the folic acid metabolism. Folic acid is the most oxidized and stable form of folate and must be deconjugated, reduced, and methylated to be metabolically active in the cell (Kelly GS, "Folates: supplemental forms and therapeutic applications"; Altern Med Rev 1998;3:208-20).

Folic acid supplementation to reduce toxicity is thus actually quite inefficient, and may additionally lead to unwanted metabolic intermediates.

Conventional methods for treating cancers usually involve combining two or more different chemotherapeutic agents. However, due to the severe side-effects, the combination possibilities has up to now been considerably limited. There is thus a need for a way of reducing toxicity, and at the same time maintining or improving the efficiency of chemotherapeutic agents, in order to be able to use chemotherapeutic substances efficiently in clinic.

Summary of the invention

An aim of the present invention is to overcome the above-mentioned drawbacks and provide a way of reducing the toxicity, and maintaining or improving the efficiency, of chemotherapeutic drugs.

This aim is achieved by the use of tetrahydrofolate (THF), methylene-tetrahydrofolate (methylene-THF) and/or methyl-tetrahydrofolate (methyl-THF), or isomers thereof, and at least one chemotherapeutic agent selected from the group consisting of multi-targeting antifolates, anthracyclines, platinum derivatives, topoisomerase inhibitors, and antimetabolites for the manufacture of a pharmaceutical composition for the treatment of cancer.

The use of THF, methylene-THF and/or methyl-THF in combination with chemotherapeutic agents according to the present invention dramatically reduces the side effects of chemotherapeutic agents and, consequently, the therapeutic index is improved. It is thus possible to administer a higher dose to the tumor without increasing the side-effects, which leads to a better clinical effect.

Said multi-targeting antifolate is selected from the group consisting of premetrexed, raltitrexed, and lometrexol; said anthracycline is selected from the group consisting of doxorubicin and epirubicin; said platinum derivative is selected from the group consisting of oxaliplatin, cisplatin, and carboplatin; said topoisomerase inhibitor is selected from the group consisting of irinotecan and CPT11; and said antimetabolite is selected from the group consisting of capecitabine, gemcitabin, UFT and S1. However, also other substances belonging to the aforementioned groups of substances may be used.

The THF, methylene-THF and/or methyl-THF may be in the form of a racemic mixture containing 50% R-configuration and 50% S-configuration. However, the proportion may vary, e.g. a mixture containing 80% R-configuration and 20% S-configuration could be used, or a mixture containing 100% R-configuration and 0% S-configuration could

be used. Ideally, an optimal dose is determined based on a patient's individual need.

The THF, methylene-THF and/or methyl-THF or isomers thereof and said chemotherapeutic agents may be formulated in different pharmaceutical compositions, or in a common pharmaceutical composition. The formulation into different compositions provides for a great administration flexibility. The formulation into a common pharmaceutical composition, on the other hand, provides for a simple manufacturing process, as well as for a simple way of administration.

Examples of cancers to be treated according to the invention are breast cancer, gastric cancer, gall bladder cancer, bile duct cancer, colon cancer, rectal cancer, liver cancer, pancreatic cancer, head and neck cancer, and mesotheolioma cancer.

The present invention also relates to a pharmaceutical composition comprising at least one chemotherapeutic agent selected from the group consisting of multi-targeting antifolates, anthracyclines, platinum derivatives, topoisomerase inhibitors, and antimetabolites, and THF, methylene-THF and/or methyl-THF or isomers thereof.

Further, the present invention relates to a kit comprising a pharmaceutical composition comprising at least one chemotherapeutic agent selected from the group consisting of multi-targeting antifolates, anthracyclines, platinum derivatives, topoisomerase inhibitors, and antimetabolites and a pharmaceutical composition comprising THF, methylene-THF and/or methyl-THF or isomers thereof.

The present invention also relates to a method for the treatment of cancer comprising administering to a patient a pharmaceutically active amount of at least one chemoterapeutic agent selected from the group consisting of multi-targeting antifolates, anthracyclines, platinum derivatives, topoisomerase inhibitors, and antimetabolites and a pharmaceutically active amount of THF, methylene-THF and/or methyl-THF or isomers thereof.

Brief description of the drawings

Fig 1 shows the main metabolic pathways by which folate and homocysteine impact on DNA synthesis, repair and methylation.

Fig 2 is a scheme showing how the anticancer drug anthracycline can form covalent adducts with DNA after intercalation.

Fig 3 shows the percent increase of tissue concentration of methylenetetrahydrofolate (MTHF) in liver metastases from colorectal cancer in individual patients.

Fig 4 shows the mean percent increase of tissue concentration of methylenetetrahydrofolate (MTHF) in liver metastases from colorectal cancer in individual patients.

Detailed description of the invention

In the research work leading to the present invention, the inventors surprisingly found that by co-administering methylene-THF or methyl-THF and chemotherapeutic agents, such as multi-targeting antifolates, anthracyclines, platinum derivaties, topoisomerase inhibitors, and antimetabolitess, it is possible to remarkably reduce toxic side-effects without diminishing the anti-tumour action of the drugs.

5,10-methylene-tetrahydrofolate (in the following referred to as methylene-THF, or CH_2FH_4) is a normal intracellular metabolite of folic acid, for use in thymidylate synthesis by thymidylate synthase (TS). The same is true with respect to the polyglutamates of methylene-THF. Methylene-THF is also used by several other enzymes including CH_2FH_4-reductase, serine hydroxymethylase and Cl-tetrahydrofolate synthase and CH_2FH_4 dehydrogenase. These interconversions using methylene-THF are essential for purine synthesis, amino acid synthesis, and lipid metabolism. Thus, methylene-THF is located at a metabolic branch point as a substrate for at least 4 different enzymes (Spears et al; US Patent no 5,376,658).

As stated above, previous studies have shown that folate status may modulate the toxicity and efficacy of cancer chemotherapy. The present inventors have found that the positive effect of folic acid is due to the increased level of methylene-THF that is formed from folic acid. Higher levels of methylene-THF in the cells leads to a more efficient TS-inhibition, which results in a thymidine-less state and increases uracil misincorporation in DNA.

Since methylene-THF is the active substance of folic acid metabolism, the use of this endogen folate for reducing toxicity in cancer treatment is much more favourable than the use of folic acid.

Dietary folates are a mixture of polyglutamated folates that are digested to monoglutamyl forms by the action of an enzyme anchored to the small intestinal brush border membrane and expressed by the glutamate carboxypeptidase II gene (GCPII) (Devlin AM et al, "Glutamate carboxypeptidase II: a polymorphism associated with lower levels of serum folate and hyperhomocysteinemia"; Hum Mol Genet 2000;9:2837-44). After deconjugation in the small intestine, folic acid is reduced to tetrahydrofolate in the liver. Reduced folates are secreted into the small intestine with bile, where they are reabsorbed and distributed to other tissues.

The reduced folate carrier, RFC-1, is the major transporter of reduced folates into the cells (Sirotnak FM, Tolner B, "Carrier-mediated membrane transport of folates in mammalian cells"; Annu Rev Nutr 1999;19:91-122). Intracellularly, reduced folate monoglutamates are converted to polyglutamates by the enzyme folylpolyglutamate synthase (FPGS) (Shane B, "Folylpolyglutamate synthesis and role in the regulation of one-carbon metabolism"; Vitam Horm 1989;45:263-335). The polyglutamated form of tetrahydrofolate is then further converted to 5,10-methylenetetrahydrofolate (methylene-THF), required as a methyl donor in the conversion of dUMP to dTMP

(Spears CP et al, "Deoxyuridylate effects on thymidylate synthase-5-fluorodeoxyuridylate- folate ternary complex formation"; Biochem Pharmacol 1989;38:2985-93; Spears CP, et al "Thymidylate synthetase inhibition in malignant tumors and normal liver of patients given intravenous 5-fluorouracil", Cancer Res 1984;44:4144-50). The conversion is catalysed by thymidylate synthase (TS). Methylene-THF is also the precursor of the metabolically active 5-methyltetrahydrofolate (methyl-THF), utilized in the re-methylation of homocysteine. Conversion of methylene-THF to methyl-THF is dependent on the enzyme methylene-tetrahydrofolate reductase (MTHFR).

The enzyme γ-glutamyl hydrolase (GGH) catalyzes the degradation of inter- and intracellular polyglutamates (Galivan J et al, "Glutamyl hydrolase. pharmacological role and enzymatic characterization", Pharmacol Ther 2000;85:207-15). Fig. 1 summarizes the main metabolic pathways by which folate and homocysteine impact on DNA synthesis, repair and methylation. As shown, proper functioning of the DNA synthesis and methylation pathways requires riboflavin (vitamin B2), pyridoxine (vitamin B6), and cobalamin (vitamin B12), in addition to folates. Inadeqate levels of any of these metabolites will result in elevated homocysteine levels. Pyridoxine deficiency will also impair the cellular ability to produce glutathione, the master antioxidant needed for detoxification of free radicals and alkylating agent damage.

Oral folates are generally available in two supplemental forms, folic and folinic acid. The biochemical basis for modulation of fluorouracil (FU) activity by folic acid or folinic acid (leucovorin) is elevation of the metabolite methylene-THF, which stabilizes the inhibitory ternary complex formed between methylene-THF, thymidylate synthase and the active metabolite of 5-FU. Folinic acid is an immediate precursor of methylene-THF, since oral administration of folinic acid bypasses the deconjugation and reduction steps needed for folic acid. Folinic acid

seems to be a more metabolically active form of folate than folic acid and, as a reduced folate, might directly cross the brain-blood barrier. Like folic acid, folinic acid must be methylated to be metabolically active. The methylation step of folic and folinic acid requires adequate levels of serine and a functional serine hydroxymethyltransferase (SHMT) enzyme (Costi MP, Ferrari S, "Update on antifolate drugs targets"; Curr Drug Targets 2001;2:135-66). Supplementation with the reduced folates methylene-THF and methyl-THF has the advantage of bypassing this methylation step.

Pharmacogenetics aims to identify individuals predisposed to high risk of toxicity from conventional doses of cancer chemotherapeutic agents. Interindividual variability in the efficacy and toxicity of drug therapy is associated with polymorphisms in genes encoding drug-metabolizing enzymes, transporters, or drug targets.

The key metabolite methylene-THF is involved directly in biosynthesis of dTMP, in conversion to 10-formyl-THF for purine synthesis, and in reduction to methyl-THF for methylation of homocysteine to methionine (Kelly GS. Folates: supplemental forms and therapeutic applications. Altern Med Rev 1998;3:208-20). The conversion of methylene-THF to methyl-THF requires the enzyme MTHFR.

Aberrations in the distribution of different folates may be caused by the MTHFR C677T polymorhism (Guinotte CL et al, "Methylenetetrahydrofolate reductase 677C-->T variant modulates folate status response to controlled folate intakes in young women"; J Nutr 2003;133:1272-80). Homo- or heterozygosity for the T allele results in a MTHFR enzyme with suboptimal activity and a raise in methylene-THF. A link between MTHFR polymorphism and tumor response to 5-FU was detected in a recent study by Cohen et al (Cohen V et al, "Methylenetetrahydrofolate Reductase Polymorphism in Advanced Colorectal Cancer: A Novel Genomic Predictor of Clinical Response to Fluoro-

pyrimidine-based Chemotherapy"; Clin Cancer Res 2003; 9:1611-5). Patients with CT or TT genotype responded better to the therapy than patients with the CC genotype. The MTHFR C677T genotype may also be predictive of clinical toxicity to raltitrexed (Stevenson JP et al "Phase I clinical and pharmacogenetic trial of irinotecan and raltitrexed administered every 21 days to patients with cancer"; J Clin Oncol 2001;19:4081-7). Homozygotes with the MTHFR C677T polymorphism experienced less raltitrexed-associated toxicity compared to those with wild-type or heterozygous genotypes.

Conversion of methylene-THF to methyl-THF may be impaired in individuals carrying a MTHFR enzyme with suboptimal activity (Guinotte CL et al "Methylenetetrahydrofolate reductase 677C-->T variant modulates folate status response to controlled folate intakes in young women"; J Nutr 2003;133:1272-80). Also, high levels of homocysteine or S-adenosylhomocysteine, as is found in folate deficient individuals, are known to inhibit the MTHFR enzyme (De Cabo SF et al, "Molecular and cytological evidence of S-adenosyl-L-homocysteine as an innocuous undermethylating agent in vivo"; Cytogenet Cell Genet 1995;71:187-92). Thus, when choosing between methylene-THF and methyl-THF it might be important to take the patients genotype and folate status into consideration.

The X-ray repair cross-complementing gene I (XRCC1) protein has an important function in base excision repair of DNA. Different polymorphic forms of the protein exist, and the Arg399Gln polymorphism has been associated with risk of developing CRC as well as with resistance to oxaliplatin/5-FU chemotherapy (Stoehlmacher J et al, "A polymorphism of the XRCC1 gene predicts for response to platinum based treatment in advanced colorectal cancer" Anticancer Res 2001;21:3075-9). Possibly, the XRCC1 gene may also be inactivated by aberrant methylation or by mutations caused by folate deficiency. Reversal of ox-

aliplatin resistance could be accomplished using folates like methylene-THF.

Further, low dietary intake or blood concentrations of folate are associated with an increased risk of colorectal neoplasia. In fact, epidemiologic studies have established an inverse association between folate status and colorectal neoplasia (Kim et al; "Colonic mucosal concentrations of folate correlate well with blood measurements of folate status in persons with colorectal polyps", Am J Clin Nutr 1998; 68: 866-72).

The use of THF, methylene-THF and methyl-THF for treatment of folate deficiency in colonic mucosa is very advantageous, since the most efficient folate is thereby administered directly to the site where it is needed the most.

In connection to the present invention, natural as well as synthetic THF, methylene-THF and methyl-THF may be used. Further, isomers of THF, methylene-THF and methyl-THF may be used. By isomers of THF, methylene-THF and methyl-THF is meant e.g. the stereoisomers of the aforementioned substances. Further, prodrugs to THF, methylene-THF and methyl-THF may be used.

A racemic mixture containing 50% R-configuration and 50 % S-configuration is suitable for use according to the invention. By a racemic mixture is meant an equimolar mixture of two enantiomers. The (R-S) system, or the Cahn-Ingold-Prelog system, is widely used and is part of the IUPAC rules. The biologically active form of THF, methylene-THF and methyl-THF is the R-configuration.

Alternative expressions for metylene-THF are 5,10-methylene-tetrahydrofolate, (6R,S)-methylene-tetrahydro folic acid, CH_2FH_4 or 5,10-methylenetetrahydropteroyl-glutamic acid (5,10-CH_2-H_4-PteGlu)

The term "chemotherapeutic agent" as used herein relates to a medicament for the treatment of cancer.

The term "multitargeting antifolate" as used herin relates to an antifolate which acts on two or more of the

enzymes involved in folate synthesis. The difference between single enzyme targeting antifolates and multitargeting antifolates is illustrated in D1, where the inhibitory activity of pemetrexed and MTX against different enzymes is compared. Pemetrexed demonstrate significant inhibitory activity for multiple enzyme systems, unlike MTX.

The terms "anthracycline", "platinum derivative", "topoisomerase inhibitor", and "antimetabolite" as used herein relates to these compounds as defined in the National Library of Medicine.

The term "patient" as used herein relates to any human or non-human mammal in need of being treated with the methods, kit or pharmaceutical compositions according to the invention.

The term "treatment" as used herein relates to both treatment in order to cure or alleviate the symptoms of different types of cancer, and to treatment in order to prevent the development of cancer. In particular, solid tumors are well suited to be treated according to the invention.

The term "pharmaceutically active amount" as used herein relates to a dose of a substance that will lead to the desired pharmacological and/or therapeutic effect. The desired pharmacological and/or therapeutic effect is, as stated above, to cure or alleviate the symptoms of different types of cancer, and to prevent the development of cancer.

The THF, methylene-THF and/or methyl-THF and the chemotherapeutic agents may be administered simultaneously or consequtively. When administered consequtively, either the THF, methylene-THF and/or methyl-THF is administered first and thereafter the chemotherapeutic agents, or the chemotherapeutic agents are administered first and thereafter the THF, methylene-THF and/or methyl-THF. The interval between the administrations depend on the drug

characteristics, and may e.g. vary from hours to days. However, shorter and longer intervals may be used.

The order in which drugs and folate are administered could be of major importance for the outcome of chemotherapeutic treatment. As an illustration, Raghunathan et al., showed that tumor growth was suppressed approximately 80% when 5-FU was administered to folate depleted animals 1 hr after folinic acid administration, compared with approximately 50% suppression in control mice (Raghunathan K et al "Impact of schedule on leucovorin potentiation of fluorouracil antitumor activity in dietary folic acid deplete mice"; Biochem Pharmacol 1997;53:1197-202). Folinic acid administered 12 hr before 5-FU resulted in tumor growth stimulation that was consistent with the pronounced growth stimulation when folinic acid was administered without 5-FU.

The pharmaceutical compositions according to the invention may also comprise other substances, such as an inert vehicle, or pharmaceutical acceptable adjuvants, carriers, preservatives, ascorbic acid, ascorbate, antioxidants, etc, which are well known to persons skilled in the art.

The pharmaceutical compositions according to the invention may be formulated by conventional manufacturing methods, such as e.g. by manufacturing methods similar to those used for the production of leucovorin.

Examples of cancers to be treated according to the invention are breast cancer, gastric cancer, gall bladder cancer, bile duct cancer, colon cancer, rectal cancer, liver cancer, pancreatic cancer, head and neck cancer, and mesotheolioma cancer.

The THF, methylene-THF and/or methyl-THF is preferably administered in a dose of 1 mg to 1000 mg, preferably a dose of 100-200 mg, corresponding to approximately 1-5 µg/kg body weight. The dose may be administered e.g. daily, weekly, or monthly. It may be administered subcu-

taneously, intramuscularely, intravenously, intraarterially, intraperitoneally, intranasally or orally.

Furthermore, it is possible to combine the treatment according to the invention with other conventional pharmacological treatments of cancer. THF, methylene-THF and/or methyl-THF may thus be administered in combination with other conventional pharmaceuticals used to treat cancer.

The compositions according to the invention may also be co-administererd with vitamin B12, vitamin B6, and vitamin B2.

Examples of combinations of chemotherapeutic agents and THF, methylene-THF and/or methyl-THF are:

- Premetrexed + THF, methylene-THF and/or methyl-THF
- Premetrexed + 5-fluorouracil + THF, methylene-THF and/or methyl-THF
- Raltitrexed + THF, methylene-THF and/or methyl-THF
- Raltitrexed + 5-fluorouracil + THF, methylene-THF and/or methyl-THF
- Capecitabine + oxaliplatin + THF, methylene-THF and/or methyl-THF
- Oxaliplatin + 5-fluorouracil + THF, methylene-THF and/or methyl-THF
- Oxaliplatin + CPT11 + 5-fluorouracil + THF, methylene-THF and/or methyl-THF
- CPT11 + 5-fluorouracil + THF, methylene-THF and/or methyl-THF.

Examples

The invention will now be further illustrated by way of examples. The examples should in no way be construed as limiting the scope of the invention.

Example 1: Multifunctional antifolates

Methylated folates may reduce severe diarrhea induced e.g. by pemetrexed (trade name: Alimta) by protecting the neural network in epithelial mucosa. Neural cells in the mucosa may be required for excretion of mucus from goblet cells.

Administration of THF, methylene-THF and/or methyl-THF also reduces side-effects from raltitrexed. The multifunctionality of raltitrexed is shown e.g. by Jackman AL et al in "ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth in vitro and in vivo: a new agent for clinical study", Cancer Res 1991 Oct 15; 51(20):5579-86.

Example 2: Anthracyclines

The anticancer drug anthracycline can form covalent adducts with DNA after intercalation (Fig. 2), and the magnitude of these adducts seems to be limited by the cellular availability of formaldehyde (Cutts SM et al, "Sequence specificity of adriamycin-DNA adducts in human tumor cells", Mol Cancer Ther 2003;2:661-70).

The anthracycline doxorubicin (trade name: Adriamycin) in combination with the anticancer agent pivaloyloxymethyl butyrate (AN-9) enhanced levels of doxorubicin-DNA adducts when treatment combinations known to be synergistic were used and were diminished using treatments known to be antagonistic.

The relative timing of the addition of doxorubicin and AN-9 was critical, with a 20-fold enhancement of doxorubicin-DNA adducts occurring when AN-9 was administered 2 h after the exposure of cells to doxorubicin. The

enhanced levels of these adducts and the accompanying decreased cell viability were directly related to the release of formaldehyde from AN-9, providing evidence for the formaldehyde-mediated activation of doxorubicin (Cutts SM et al "Molecular basis for the synergistic interaction of adriamycin with the formaldehyde-releasing prodrug pivaloyloxymethyl butyrate (AN-9)"; Cancer Res 2001;61:8194-202). Since methylene-THF is known to be unstable and dissociates spontaneously into formaldehyde and tetrahydrofolate in cells, treatment with anthracyclines and methylene-THF is also synergistic.

Example 3: Platinum derivatives

Methyl donation to neural membrane lipids is important for neurological function and neurotransmission. Folate deficiency leads to disruption of myelin which may cause polyneuropathy (Di Trapani et al, "Dementia-peripheral neuropathy during combined deficiency of vitamin B12 and folate. Light microscopy and ultrastructural study of sural nerve"; Ital J Neurol Sci 1986;7:545-52). Thus, supplementation with methylated folates like methylene- or methyltetrahydrofolate reduces polyneuropathy, a common side-effect caused by several cytotoxic drugs, especially oxaliplatin.

Example 4: Topoisomerase inhibitors

Methylated folates also reduces severe diarrhea induced e.g. by irinotecan (trade name: Campto) by protecting the neural network in epithelial mucosa. Neural cells in the mucosa may be required for excretion of mucus from goblet cells.

Another example of a topoisomerase inhibior is CPT11, which is a topoisomerase I-inhibitor.

Example 5: Antimetabolites

The antimetabolite (prodrug) capecitabine (trade name: Xeloda) is converted to 5-FU by the enzyme

thymidine phosphorylase (TP) (Miwa M et al, "Design of a novel oral fluoropyrimidine carbamate, capecitabine, which generates 5-fluorouracil selectively in tumours by enzymes concentrated in human liver and cancer tissue"; Eur J Cancer 1998;34:1274-81). It is thought that capecitabine is most effective in tumors with high TP levels since TP degrades endogenous thymidine, increasing the effects of TS-inhibition.

Capecitabine in combination with methylene-THF is synergistic since tumors and normal tissue with a high TP have an increased degradation of endogenous thymidine. Thus, lack of thymidine in the cells would be predicted to result in a high consumption of methylenetetrahydrofolate for DNA synthesis and repair, affecting the methylation pathway negatively (conversion of methylene-THF to methyl-THF). Supplementation with folate during treatment with capecitabine may increase therapeutic efficacy and reduce side-effects caused by deficient methylation. Administration with methylene-THF or methyl-THF instead of folinic acid is more efficient since several reduction and methylation steps can be omitted.

Example 6

The level of increase of tissue concentration of methylene-THF after administration of leucovorin and methylene-THF, respectively, has been compared.

Patients operated upon due to liver metastases were given a dose of folates. Biopses were taken from the tumor before i.v. bolus injection of leucovorin on methylenetetrahydrofolate. At 20 minutes a new tumor biopsy was taken and the concentration of MTHF was assayed. The administration of methylenete-THF led to a greater tissue concentration of methylene-THF than leucovorin. The results are shown in figs 3 and 4.

Methods

Determination of folate and homocysteine levels

Erythrocyte folate, serum folate, and serum homocysteine is determined by standard HPLC techniques (Garcia A, Apitz-Castro R, "Plasma total homocysteine quantification: an improvement of the classical high-performance liquid chromatographic method with fluorescence detection of the thiol-SBD derivatives"; J Chromatogr B Analyt Technol Biomed Life Sci 2002;779:359).

Determination of folate by the FdUMP-binding assay

The FdUMP-binding assay is used for measuring the folate levels in the tissues. Total reduced folate and methylene-THF concentrations is analyzed using the Priest methodology, (Priest DG et al, "Relationship of reduced folate changes to inhibition of DNA synthesis induced by methotrexate in L1210 cells in vivo"; Cancer Res 1989;49:4204-9), slightly modified (Spears CP, Gustavsson BG, "Methods for thymidylate synthase pharmacodynamics: serial biopsy, free and total TS, FdUMP and dUMP, and H4PteGlu and CH2-H4PteGlu assays"; Adv Exp Med Biol 1988;244:97-106).

Gene expression studies

Quantitative gene expression of the folate-associated genes RFC, FPGS, GGH, TS, and MTHFR is determined using real-time PCR analysis of cDNA obtained from micro-dissected, paraffin-embedded intestinal normal mucosa and malignant tumor. Total RNA is isolated according to Chomczynski and Sacchi (Chomczynski P, Sacchi N, "Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction"; Anal Biochem 1987;162:156-9) and reverse-transcribed according to Horikishi et al (Horikoshi T et al, "Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in human tumors using the po-

lymerase chain reaction"; Cancer Res 1992;52:108-1). Quantitative PCR is performed using the Sequence Detector ABI Prism 7700 (Applied Biosystems).

Analysis of hypermethylated DNA sequences

Microdissected, bisulfite-treated genomic DNA is extraxted from normal and malignant tissues. Unmethylated cytosines in DNA is chemically modified to uracil using bisulfite-treatment whereas unmethylated cytosines remain unaffected by the process. Bisulfite-treated DNA is then quantified using a fluorescence-based real-time PCR method (Trinh BN et al, "DNA methylation analysis by MethyLight technology"; Methods 2001;25:456-62). Specific primers and probes amplifying the genes APC, p16, hMLH1 and MYOD1 (internal reference) are used.

Microsatellite instability

Five microsatellites (D2S123, D5S346, D17S250, BAT25, and BAT26) is analysed in genomic DNA from microdissected normal and malignant tissues using capillary electrophoresis and fragment analysis with fluorescent primers (Odin E et al "Rapid method for relative gene expression determination in human tissues using automated capillary gel electrophoresis and multicolor detection"; J Chromatogr B Biomed Sci Appl 1999;734:47-53).

Analysis of polymorphic markers using large-scale genomic sequencing

Blood samples is analyzed at the SweGene Gothenburg Resource Center, platform PPD (profiling polygenetic diseases). The line from whole blood sample to genotype is highly automated and high-throughput analyses for the genotyping of clinical material will be used. Genomic DNA from blood and tissue will be analyzed by real-time PCR using fluorescent primers and probes (Ulvik A, Ueland PM, "Single nucleotide polymorphism (SNP) genotyping in unprocessed whole blood and serum by real-time PCR: appli-

cation to SNPs affecting homocysteine and folate metabolism"; Clin Chem 2001;47:2050-3). The markers to be genotyped include MTHFR, MS, GCPII, and XRCC1.

CLAIMS

1. Use of tetrahydrofolate, methylene-tetrahydrofolate and/or methyl-tetrahydrofolate, or isomers thereof, and at least one chemotherapeutic agent selected from the group consisting of multi-targeting antifolates, anthracyclines, platinum derivatives, topoisomerase inhibitors, and antimetabolites for the manufacture of a pharmaceutical composition for the treatment of cancer.

2. Use according to claim 1, wherein said multi-targeting antifolate is selected from the group consisting of premetrexed, raltitrexed, and lometrexol.

3. Use according to claim 1, wherein said anthracycline is selected from the group consisting of doxorubicin and epirubicin.

4. Use according to claim 1, wherein said platinum derivative is selected from the group consisting of oxaliplatin, cisplatin, and carboplatin.

5. Use according to claim 1, wherein said topoisomerase inhibitor is selected from the group consisting of irinotecan and CPT11.

6. Use according to claim 1, wherein said antimetabolite is selected from the group consisting of capecitabine, and gemcitabin.

7. Use according to any one of the preceding claims, wherein said tetrahydrofolate, methylene-tetrahydrofolate and/or methyl-tetrahydrofolate is in the form of a racemic mixture containing 50% R-configuration and 50% S-configuration.

8. Use according to any one of the preceding claims, wherein said tetrahydrofolate, methylene-tetrahydrofolate and/or methyl-tetrahydrofolate, or isomers thereof, and said at least one chemotherapeutic agent are formulated in different pharmaceutical compositions.

9. Use according to any one of the preceding claims, wherein said tetrahydrofolate, methylene-tetrahydrofolate

and/or methyl-tetrahydrofolate, or isomers thereof, and said at least one chemotherapeutic agent are formulated in a common pharmaceutical composition.

10. Use according to any one of the preceding claims wherein said cancer is selected from the group consisting of breast cancer, gastric cancer, gall bladder cancer, bile duct cancer, colon cancer, rectal cancer, liver cancer, pancreatic cancer, head and neck cancer, and mesotheolioma cancer.

11. A pharmaceutical composition comprising tetrahydrofolate, methylene-tetrahydrofolate and/or methyl-tetrahydrofolate, or isomers thereof, and at least one chemotherapeutic agent selected from the group consisting of multi-targeting antifolates, anthracyclines, platinum derivatives, topoisomerase inhibitors, and antimetabolites.

12. A pharmaceutical composition according to claim 11, wherein said multi-targeting antifolate is selected from the group consisting of premetrexed, raltitrexed, and lometrexol.

13. A pharmaceutical composition according to claim 11, wherein said anthracycline is selected from the group consisting of doxorubicin and epirubicin.

14. A pharmaceutical composition according to claim 11, wherein said platinum derivative is selected from the group consisting of oxaliplatin, cisplatin, and carboplatin.

15. A pharmaceutical composition according to claim 11, wherein said topoisomerase inhibitor is selected from the group consisting of irinotecan and CPT11.

16. A pharmaceutical composition according to claim 11, wherein said antimetabolite is selected from the group consisting of capecitabine, and gemcitabin.

17. A pharmaceutical composition according to any one of the claims 11-16, wherein said tetrahydrofolate, methylene-tetrahydrofolate and/or methyl-tetrahydrofolate

is in the form of a racemic mixture containing 50% R-configuration and 50% S-configuration.

18. A pharmaceutical composition according to any one claims 11-17, wherein said tetrahydrofolate, methylene-tetrahydrofolate and/or methyl-tetrahydrofolate, or isomers thereof, and said at least one chemotherapeutic agent are formulated in different pharmaceutical compositions.

19. A pharmaceutical composition according to any one of the claims 11-17, wherein said tetrahydrofolate, methylene-tetrahydrofolate and/or methyl-tetrahydrofolate, or isomers thereof and said at least one chemotherapeutic agent are formulated in a common pharmaceutical composition.

20. A kit comprising a pharmaceutical composition comprising a pharmaceutical composition comprising tetrahydrofolate, methylene-tetrahydrofolate and/or methyl-tetrahydrofolate, or isomers thereof, and at least one chemotherapeutic agent selected from the group consisting of multi-targeting antifolates, anthracyclines, platinum derivatives, topoisomerase inhibitors, and antimetabolites.

21. A method for the treatment of cancer comprising administering to a patient a pharmaceutically active amount of tetrahydrofolate, methylene-tetrahydrofolate and/or methyl-tetrahydrofolate, or isomers thereof, and a pharmaceutically active amount of at least one chemoterapeutic agent selected from the group consisting of multi-targeting antifolates, anthracyclines, platinum derivatives, topoisomerase inhibitors, and antimetabolites.

22. A method according to claim 21, wherein said multi-targeting antifolate is selected from the group consisting of premetrexed, raltitrexed, and lometrexol.

23. A method according to claim 21, wherein said anthracycline is selected from the group consisting of doxorubicin and epirubicin.

24. A method according to claim 21, wherein said platinum derivative is selected from the group consisting of oxaliplatin, cisplatin, and carboplatin.

25. A method according to claim 21, wherein said topoisomerase inhibitor is selected from the group consisting of irinotecan and CPT11.

26. A method according to claim 21, wherein said antimetabolites is selected from the group consisting of capecitabine, and gemcitabin.

27. A method according to any one of the claims 21-26, wherein said tetrahydrofolate, methylene-tetrahydrofolate and/or methyl-tetrahydrofolate, or isomers thereof, and said at least one chemotherapeutic agent are administered consecutively.

28. A method according to any one of the claims 21-26, wherein said tetrahydrofolate, methylene-tetrahydrofolate and/or methyl-tetrahydrofolate, or isomers thereof, and said at least one chemotherapeutic agent are administered simultaneously.

29. A method according to any one of the claims 21-28, wherein said cancer is selected from the group consisting of breast cancer, gastric cancer, gall bladder cancer, bile duct cancer, colon cancer, rectal cancer, liver cancer, pancreatic cancer, head and neck cancer, and mesotheolioma cancer.

ABSTRACT

The use of tetrahydrofolate, methylene-tetrahydrofolate and/or methyl-tetrahydrofolate, or isomers thereof, and at least one chemotherapeutic agent selected from the group consisting of multi-targeting antifolates, anthracyclines, platinum derivatives, topoisomerase inhibitors, and antimetabolites for the manufacture of a pharmaceutical composition for the treatment of cancer is disclosed. By combining the chemotherapeutic agents with tetrahydrofolate, methylene-tetrahydrofolate and/or methyl-tetrahydrofolate, or isomers thereof, it is possible to remarkably reduce toxic side-effects without diminishing the anti-tumour action of the drugs. A pharmaceutical composition, a kit comprising the pharmaceutical composition as well as a method for the treatment of cancer are also disclosed.

Fig 1

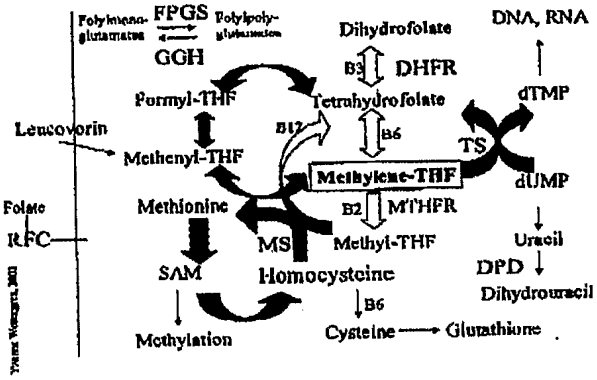

Fig. 1. The main metabolic pathways by which folate and homocysteine impact on DNA synthesis, repair and methylation. The enzyme MTHFR balances the DNA methylation and DNA synthesis pathways to maintain normal homeostasis. RFC = reduced folate carrier; FPGS = folylpolyglutamate synthase; GGH = γ-glutamyl hydrolase; TS = thymidylate synthase; THF = tetrahydrofolate; MTHFR = methylene-tetrahydrofolate reductase; MS = methionine synthase; SAM = S-adenosylmethionine; DPD = dihydro-pyrimidine dehydrogenase; B2 = riboflavin; B6 = pyridoxine; B12 = cobalamin

Fig 2

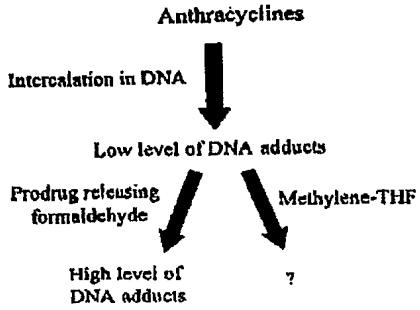

Fig 3

Percent increase of tissue concentration of methylenetetrahydrofolate (MTHF) in liver metastases from colorectal cancer in individual patients.

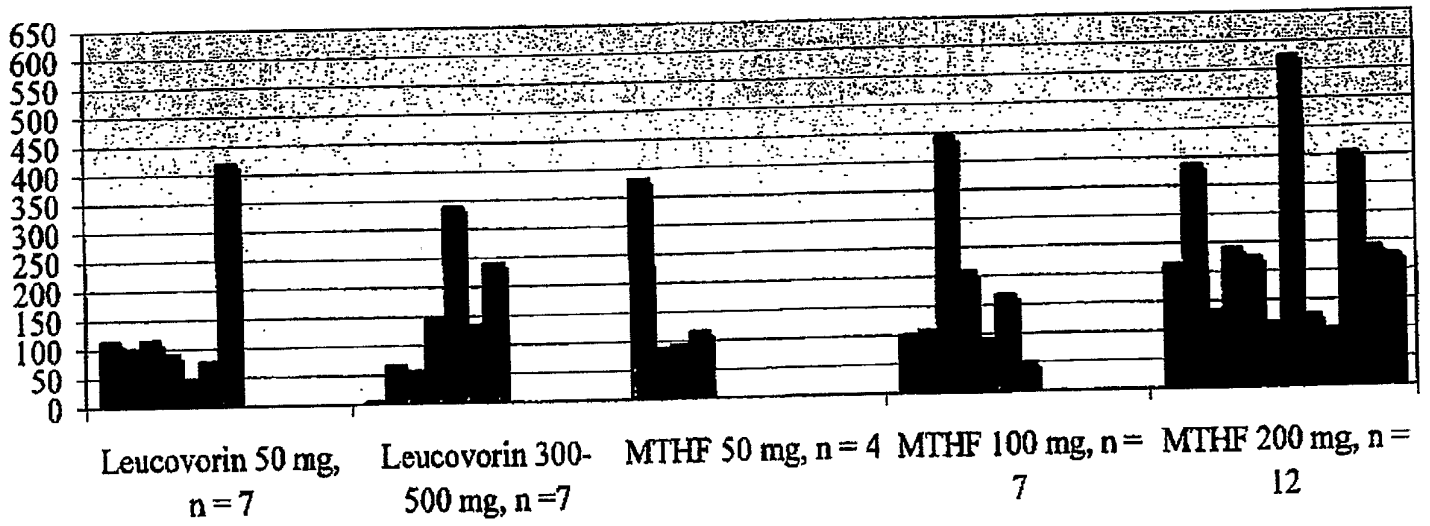

Fig 4

Mean percent increase of tissue concentration of methylenetetrahydrofolate (MTHF) in liver metastases from colorectal cancer in individual patients.